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High Performance Capillary Electrophoresis: a Fast, Cheap and Simple Method of Analysis for Determination of Individual Glucosinolates

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ABSTRACT

A high performance capillary electrophoresis (HPCE) method for determination of individual intact glucosinolates has been developed. This HPCE technique based on cetyltrimethylammonium bromide (CTAB) micellar electrokinetic chromatography successfully separated the investigated glucosinolates. It allowed efficient separation and quantitative determination of the investigated glucosinolates occurring in Brassica species including double low oilseed rape. The method has also been found well suited for determination of glucosinolates accumulated in vegetative parts of the plants. Only small amounts of glucosinolates are required for detection; pico mol amounts. It is a fast method compared to other known methods of glucosinolate analysis. For specific purposes, 5-10 min per analysis, eventually directly on the crude extracts of glucosinolates or preferably on isolated intact glucosinolates to avoid interfering peaks. The isolation procedure requires only some few minutes, when based on the technique now developed. This procedure gives also the possibilities of an efficient sample preparation, purification and concentration step. The HPCE analysis requires only some few mL of a cheap aqueous run buffer for several analyses. The capillary tube can be used for several hundreds of analyses without destruction of the tube.

INTRODUCTION

Glucosinolates are plant products with well defined structures, although appreciable structural variations are found among the more than 100 known glucosinolates (1). The glucosinolates and glucosinolate degradation products are involved in off-flavour, antinutritive and toxic effects associated with too high concentrations of these compounds in food and feed (2,3). To reduce the problems much efforts have been placed on production of high quality double low oilseed rape (4) and development of gentle methods of rapeseed processing (5). The possibilities of success in research, development and control directed at improved quality of kale, rapeseed and products thereof depend, however, on available reliable, fast, cheap and sufficiently simple methods of analysis. Limitations and possibilities of available methods have been thoroughly treated in recent years (1,6,7). It is found, that high performance liquid chromatography (HPLC) has several

advantages compared to other methods (8,9). HPLC is in consequence thereof recommended as official reference method (10) in spite of some drawbacks (1). To various purposes needs of a more simple, fast and cheap method of glucosinolate analysis are still existing.

Traditional high voltage paper electrophoretic separation of glucosinolates is a well known technique (11) of great value for qualitative but not for quantitative analysis. In this work, the potential for HPCE (12,13) has been considered as a possible method of both qualitative and quantitative glucosinolate determinations. The experiences from the original work with HPLC of glucosinolates (8,9) with and without ion-pairing (1) and micellar electrokinetic chromatography (13) are utilized in the method now presented. Fast column purification/sample preparation of intact glucosinolates (1) and HPCE with the use of cetyltrimethylammonium bromide (CTAB) micelles are combined to a simple and cheap new method of glucosinolate analysis. Rapid HPCE separation of the glucosinolates with very narrow peak widths have been obtained. The separations at the applied pH are a result of interaction (hydrophobic and ion-pairing) of the negatively charged glucosinolates and the positively charged CTAB micelles giving differential partition of glucosinolates in the micelles as for the reversed phase HPLC method (1,8,9). The HPCE method now developed is well suited for glucosinolates accumulated both in seeds and vegetative parts of double low oilseed rape, kale and other cruciferous plants.

MATERIALS AND METHODS

The apparatus used in these studies was "Model 270 A Capillary Electrophoresis System" (Applied Biosystem, USA, 850 Lincoln Center Drive, Foster City, CA 94404). Micellar HPCE electrokinetic chromatography was performed in a 720 mm x 0.05 mm i.d. fused-silica capillar tube. The instrumental conditions included 1-second vacuum injections with sample concentrations of few nanomol per mL of each glucosinolate. Injection from the negative end of the capillary, a temperature of 30°C, an applied field strength of 20 kV, 80 μA and on-column UV detection at 235 nm at a position of 500 mm from the negative end of the capillary were used. For data processing a Shimadzu Chromatopac C-R3A (Kyoto, Japan) was used. Other apparatus, materials, chemicals and experimental procedures for the glucosinolate isolation, including the fast QMA Sep-Pak technique, were as those described previously (1).

Buffer preparation comprised stock solutions of 1) sodium tetraborate (100mM), 2) sodium

phosphate (150mM), and 3) cetyltrimethylammonium bromide (100mM, CTAB= hexadecyltrimethylammonium bromide).

The run buffer solution was composed of 4.5 mL of 1) + 5.0 mL of 2) + 12.5 mL of 3) + water to a total volume of 25mL, pH = 7.0, and resulting in the concentration of 18 mM 1), 30mM 2) and 50mM 3). The solution was filtered through a 0.45 μm membrane filter prior to use.

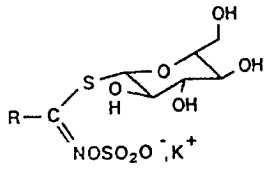
Capillary conditioning was obtained by flushing the capillary tube with run buffer for 7 min using vacuum before each run.

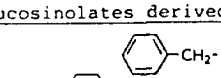
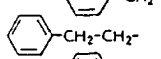
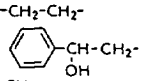
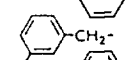
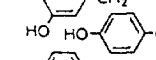
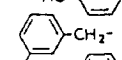
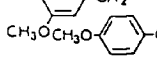
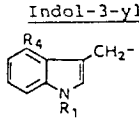
RESULTS AND DISCUSSION

Glucosinolates used in the present study were from our own collection (1). These compounds have been isolated by well documented methods (3,14) and determination of their purity and identification have been based on traditional methods (1,11,14).

Structures and names of the glucosinolates used in the present study are presented in Figure 1, together with numbers used in connection with the HPCE electropherograms. The glucosinolates used as reference compounds have been selected according to variations in structure and properties considered to be of special interest with respect to requirement of reliable analytical methods (1).

Isolation and QMA Sep-Pak purification of intact glucosinolates accumulated in both seeds and vegetative parts of single as well as double low oilseed rape have been performed by the traditionally applied method (1). Preparation of crude extracts is a standard procedure used in connection with different methods of analyses for individual glucosinolates (1,4,9,11). The following step with isolation of intact glucosinolates using the QMA Sep-Pak technique (1) is a fast procedure which only requires about 2 min. This step gives an efficient purification and concentration of the glucosinolates in the crude extracts (vide infra).



No.	Structure of R group		Semisystematic names	Trivial names
Glucosinolates derived from methionine:				
1	$CH_2=CH-CH_2-$		Allylglucosinolate	Sinigrin
2	$CH_2=CH-CH_2-CH_2-$		But-3-enylglucosinolate	Gluconapin
3	$CH_2=CH-CH_2-CH_2-CH_2-$		Pent-4-enylglucosinolate	Glucobrassicanapin
4	$CH_2=CH-\underset{\underset{OH}{\vert}}{CH}-CH_2-$		(2R)-2-Hydroxybut-3-enylglucosinolate	Progoitrin
5	-- " --		(2S)-2-Hydroxybut-3-enylglucosinolate	Epiprogoitrin
6	$CH_2=CH-CH_2-\underset{\underset{OH}{\vert}}{CH}-CH_2-$		(2R)-2-Hydroxypent-4-enylglucosinolate	Napoleiferin
7	$CH_3-S-CH_2-CH_2-CH_2-$		3-Methylthiopropylglucosinolate	Glucoibervirin
8	$CH_3-S-CH_2-CH_2-CH_2-CH_2-$		4-Methylthiobutylglucosinolate	Glucoerucin
9	$CH_3-S-CH_2-CH_2-CH_2-CH_2-CH_2-$		5-Methylthiopentylglucosinolate	Glucoberteroin
10	$CH_3-SO-CH_2-CH_2-CH_2-$	Δ	3-Methylsulphinylpropylglucosinolate	Glucoiberin
11	$CH_3-SO-CH_2-CH_2-CH_2-CH_2-$	Δ	4-Methylsulphinylbutylglucosinolate	Glucoraphanin
12	$CH_3-SO-CH_2-CH_2-CH_2-CH_2-CH_2-$	Δ	5-Methylsulphinylpentylglucosinolate	Glucoalyssin
13*	$CH_3-SO-CH=CH-CH_2-CH_2-$	o Δ	4-Methylsulphinylbut-3-enylglucosinolate	Glucoraphenin
14	$CH_3-SO_2-CH_2-CH_2-CH_2-$		3-Methylsulphonylpropylglucosinolate	Glucocheirolin
15	$CH_3-SO_2-CH_2-CH_2-CH_2-CH_2-$		4-Methylsulphonylbutylglucosinolate	Glucoerysolin
Glucosinolates derived from phenylalanine:				
16			Benzylglucosinolate	Glucotropaeolin
17			Phenethylglucosinolate	Gluconasturtiin
18*		□	2-Hydroxy-2-phenylethylglucosinolate	Glucobarbarin
19			m-Hydroxybenzylglucosinolate	Glucolepigramin
20			p-Hydroxybenzylglucosinolate	Sinalbin
21			m-Methoxybenzylglucosinolate	Glucolimnanthin
22			p-Methoxybenzylglucosinolate	Glucoaubrietin
Indol-3-ylmethylglucosinolates biosynthetically derived from tryptophan:				
23		(R_1=R_4=H	Indol-3-ylmethylglucosinolate	Glucobrassicin
24		R_1=OCH_3; R_4=H	N-Methoxyindol-3-ylmethylglucosinolate	Neoglucobrassicin
25		R_1=SO_3^-; R_4=H	N-Sulphoindol-3-ylmethylglucosinolate	Sulphoglucobrassicin
26		R_1=H; R_4=OH	4-Hydroxyindol-3-ylmethylglucosinolate	4-Hydroxyglucobrassicin
27		R_1=H; R_4=OCH_3	4-Methoxyindol-3-ylmethylglucosinolate	4-Methoxyglucobrassicin

*Occur also as cinnamoylderivatives
Δ(R)-configuration at the sulphinyl group.
o trans-E-configuration.
□ (2S)-configuration in glucobarbarin and (2R)-configuration in glucosibarin.

Figure 1. Structures and names of the glucosinolates used in HPCE analyses. Numbers are used in connection with the electropherograms.

Figure 2 gives a schematic illustration of the CTAB micelles in the applied capillary tube, and thereby the basis for the HPCE used for the glucosinolate analysis now described.

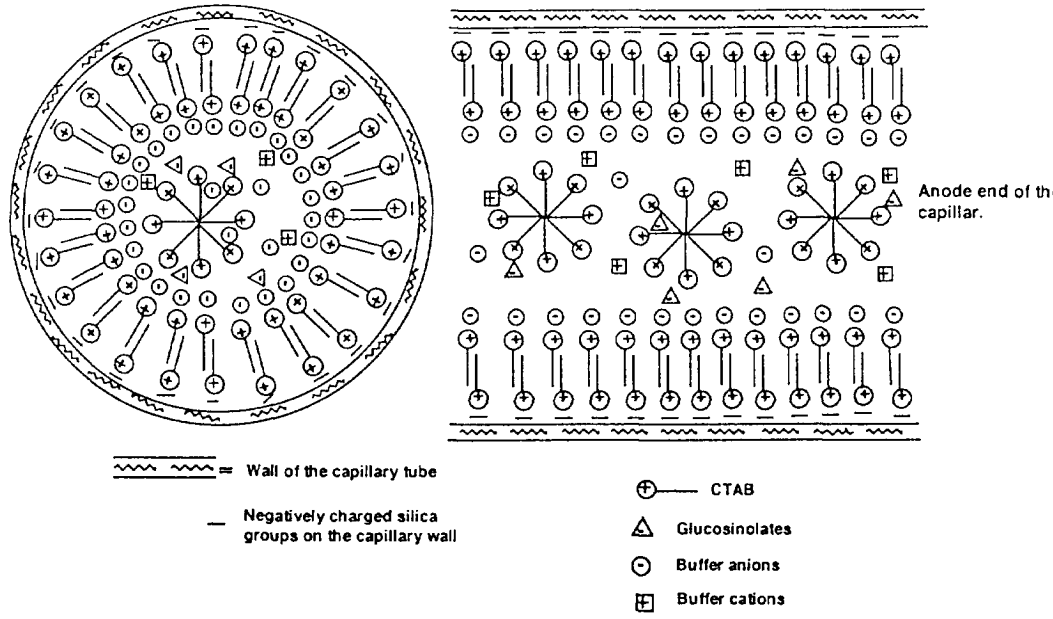


Figure 2. Illustration of the proposed conditions for the buffer ions, CTAB micelles and glucosinolates in the capillar used for HPCE of glucosinolates.

The pH of the buffer creates negatively charged silica groups on the capillary wall. This gives possibilities of CTAB to form a double layer associating one positive end to the capillary wall and the other to anions from the run buffer. CTAB also forms micelles, and glucosinolates as ion-pair with CTAB will thus have possibilities of partition between the hydrophobic CTAB micelles or double layer and the aqueous run buffer. This corresponds to the separation principles in reversed phase HPLC and SPE (solid phase extraction) techniques used in glucosinolate analysis (8-10,14). The applied temperature, run buffer and voltage over the capillary tube, with cathode at injection and anode at detection end of the capillar produces an electroosmotic flow toward the anode. Micelles with positive charges move toward the cathode with a speed depending on electroosmotic flow. The electrophoretic mobility of the glucosinolates is thus increased owing to the effect from the elctroosmotic flow but retarded owing to the flow of CTAB micelles. The glucosinolates will be in an equilibrium distribution between the two mobile phases, the hydrophobic CTAB phase and the hydrophilic buffer phase. This gives separation possibilities for the glucosinolates as in reversed phase HPLC (9) but based on other forces in the HPCE method. The HPCE method briefly described in this paper is presented with details elsewhere (Michaelsen and Sørensen in prep.).

Pure glucosinolates and mixtures of glucosinolates were analysed in order to establish the separation and elution pattern using HPCE. In addition to the glucosinolates shown in figure 1 the following glucosinolates were also used: methylglucosinolate (No. 28), 2-hydroxyisobutyl (No. 29), glucosibarin (No. 30, epimer of glucobarbarin), σ-hydroxybenzylglucosinolate (No.31, σ-isomer of glucolepigramin and sinalbin) and σ-rhamnopyranosyloxybenzylglucosinolate (No. 32, rhamnoside of No. 31).

Figure 3 shows electropherograms of reference mixtures of glucosinolates with quite different structures. The HPCE conditions were as described in materials and methods, except for the electropherogram (D). This electropherogram was obtained at 60°C. It is revealed from these electropherograms that efficient separations of glucosinolates are possible by the developed HPCE method. Even minor differences in structures are sufficient for separation, and the elution order corresponds well to the elution order of glucosinolates using reversed phase HPLC chromatography of intact compounds (1,8,9). This is in agreement with the proposed principles of separation. The time of analysis can be reduced to some few minutes with appropriate changes of the HPCE conditions, but this results of course in a more narrow time window within which separation of glucosinolates with minor differences in structures must occur.

Crude extracts and QMA eluates with glucosinolates from seeds and vegetative parts of single low and double low rapeseed cultivars were used for the electropherograms of intact glucosinolates shown in figure 4. Figure 4A shows the electropherogram of the QMA eluate, whereas figure 4B shows the electropherogram obtained by use of the crude extract from the same sample of rapeseed. The HPCE results shown in figs. 4C and 4D are obtained by use of QMA extracts from seeds and leaves, respectively, of a double low rapeseed variety with a very low level (3 μmol/g seed) of glucosinolates. Samples with very low levels of glucosinolates can give some problems with correct peak identification, if crude extracts are used directly to HPCE analyses owing to various peaks in the electropherograms from interfering non-glucosinolate peaks. Analysing samples containing very low levels of glucosinolates, it is furthermore recommendable to evaporate the QMA eluates to a reduced volume before HPCE analysis, as this new method of analysis only require small amounts of glucosinolates in few nL injection volume, but at least the same concentration as used in traditional HPLC.

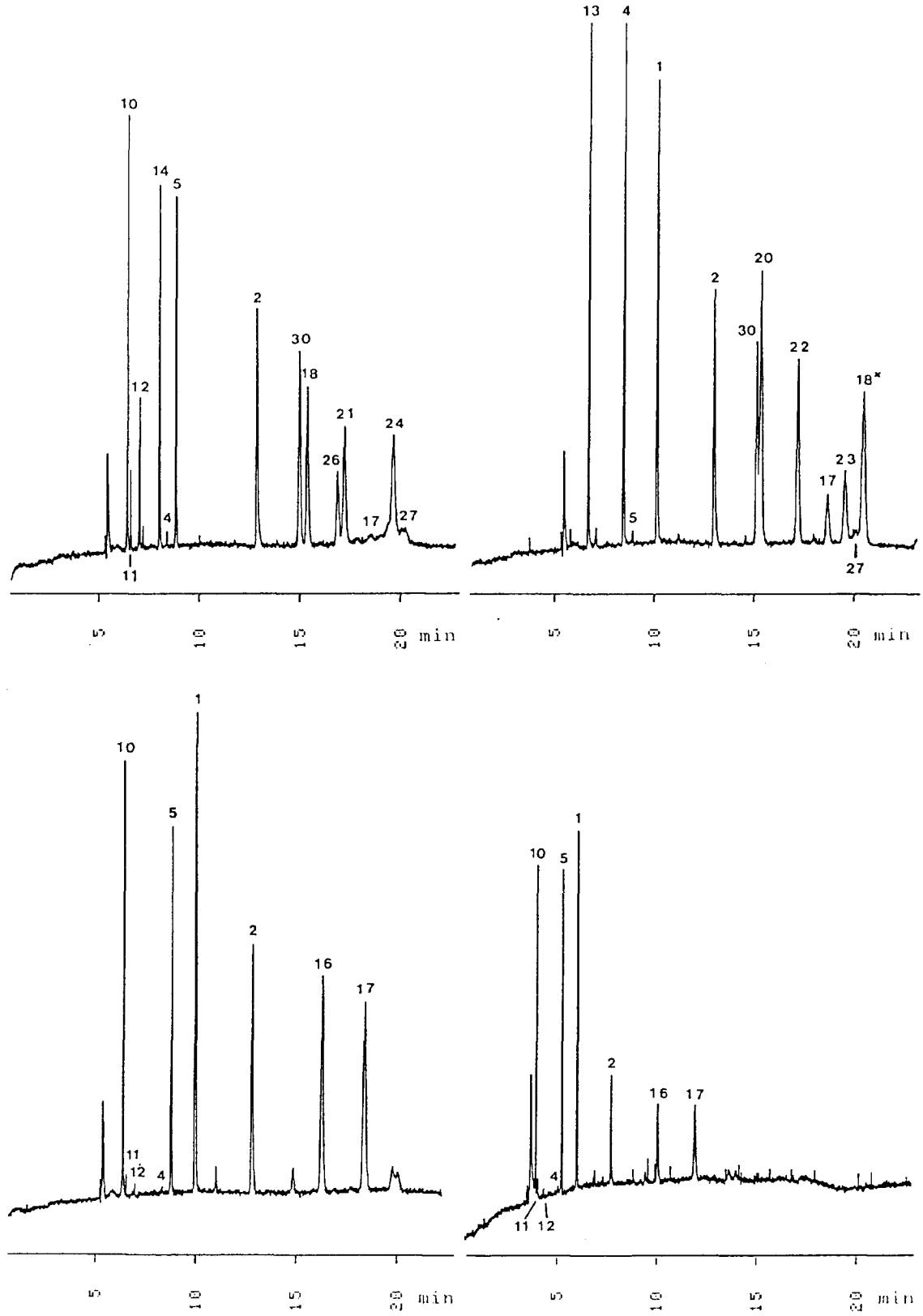


Figure 3. Electropherograms of 3 different mixtures of glucosinolates. A-C: HPCE conditions se materials and methods, D: equal conditions except for a temperature of 60°C.

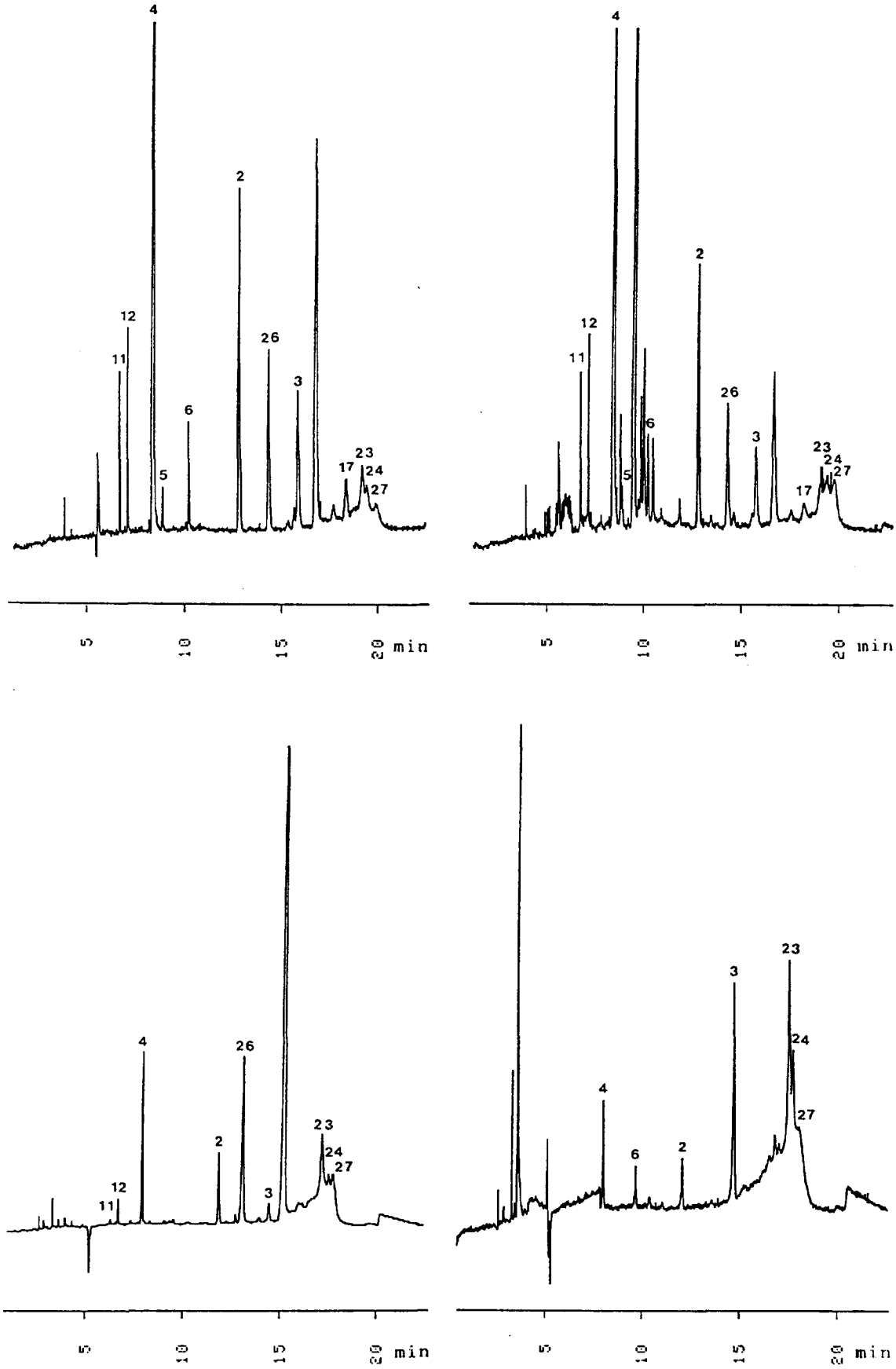


Figure 4. Electropherograms of different rape samples. A: QMA eluate and B: crude extract from seeds of a single low rapeseed variety, C: QMA eluate from seeds of a double low variety, D: QMA eluate from leaves of double low variety.

CONCLUSIONS

The HPCE method of glucosinolate analysis now described has a high separation capacity compared to other methods of analysis for determination of individual glucosinolates, e.g. HPLC (9). The method is also well suited for quantitative analysis. The capillar to HPCE is cheap compared to HPLC columns, the capillar is not sensitive to impurities as the case is for HPLC columns, and HPCE requires only very small amounts (μL) of a relatively cheap run buffer per analysis. Furthermore changes in buffers and separation conditions are very easy and fast to do. The purification of crude extracts using the QMA Sep-Pak technique is generally recommendable, and this is also a cheap and fast step (2-3 minutes). As the time for HPCE analysis is relatively short (10-20 minutes) and can be reduced to only few minutes for specific and well defined samples, the total procedure is fast compared to other known methods for determination of individual glucosinolates. The HPCE method is thus a valuable supplement to other methods of glucosinolate analysis.

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